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(57) Abstract

The present invention provides a method for producing desired proteins or chemicals in fungal host cells which comprise modulating the nucleic acid encoding proteins associated with hyphal growth. The amino acid and nucleic acid sequence of *hbrA* is provided.

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Description

HYPHAL GROWTH IN FUNGI

Field of the Invention

The present invention generally relates to hyphal growth in fungi and in particular describes the modulation of genes associated with hyphal growth in filamentous fungi. The present invention provides methods and systems for the production of proteins and/or chemicals from filamentous fungi which comprise modulation of genes associated with hyphal growth.

Background of the Invention

While the number of fungal species described is approximately 64,000, it is estimated that over one million species exist making this a diverse group of organisms. About 90% of fungi grow in the form of a radiating system of branching hyphae known as the mycelium. This mode of growth reflects a different life style from unitary organisms such as yeasts, with distinct advantages for advancing over surfaces and penetrating substrata (Carlile, 1994, The Growing Fungus, ed. Gow, N.A.R. & Gadd, G.M., Chapman & Hall, pp.3-19). To date very few genes have been characterized which effect fungal branching. The most characterized gene is cot1 isolated from the fungus *Neurospora crassa*. Cot-1 is a temperature sensitive mutation leading to hyperbranching and the sequence, whose function is unknown, appears to encode a cAMP dependent protein kinase (Yarden et al, 1992, EMBO J. 11:2159-2166).

Filamentous fungi find industrial importance as producers of antibiotics, enzymes, fine chemicals and food (*Aspergillus*: 50 Years On (1994) vol 29, ed S.D. Martinelli & J.R. Kinghorn pp. 561-596). There remains a need in the art for improved methods of producing proteins in filamentous fungus. Filamentous fungus are also known pathogens of plants and animals. Therefore, understanding the genetic basis of fungal growth will provide insight regarding possible anti-fungal therapies.

Summary of the Invention

The present invention is based, in part, upon the discovery of *Aspergillus* genes that are associated with fungal morphology and in particular with hyphal branching. A linear relationship between the degree of hyphal branching (measured as hyphal growth unit length) and culture viscosity in the fermentor (as measured by torque exerted on the rheometer impeller) has been observed. Isolation of hyper branching fungal mutants and identification of genes associated with fungal hyper branching provides a means for modulating fungal morphology thereby providing a means for controlling viscosity and improving fermentor performance.

The present invention is also based, in part, upon the identification of an *A. nidulans* mutant for the production of HbrA (the mutant being referred to herein as HbrA2) which exhibits a hyperbranching phenotype at the restrictive temperature, 42°C. The mutation HbrA2 does not appear to affect growth of *A. nidulans* at 26°C, but results in a hyperbranching, restricted growth phenotype at 42°C. The HbrA2 mutant comprising the heterologous nucleic acid encoding the *M.meihei* protease was able to secrete the protease at 26°C. The Hbr*A2* mutant was unable to secrete the protease at 37°C but was able to secrete the endogenous alpha amylase at temperatures greater than 37°C. The present invention provides the amino acid, HbrA, and nucleic acid sequence for *hbrA* and methods for producing heterologous protein or chemicals in fungi by modulating the expression of proteins associated with hyphal growth, such as HbrA.

Accordingly, the present invention provides an isolated protein associated with hyphal growth in fungi having at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the amino acid sequence as disclosed in SEQ ID NO:2. In one embodiment, the protein associated with hyphal growth is HbrA which has the amino acid sequence as disclosed in SEQ ID NO:2. The present invention provides polynucleotides encoding the amino acid having the sequence as shown in SEQ ID NO:2 as well as polynucleotides having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the polynucleotide having the sequence as shown in SEQ ID NO: 1. In one embodiment, the polynucleotide is capable of hybridizing to the polynucleotide having the sequence as shown in SEQ ID NO:1 under conditions of intermediate to high stringency, or is complementary to the polynucleotide having the sequence as shown in SEQ ID NO:1. In another embodiment, the polynucleotide has the nucleic acid sequence as disclosed in SEQ ID NO:1. The present invention also provides host cells and expression vectors comprising a polynucleotide encoding SEQ ID NO:2

In one embodiment, the host cell is a fungus and in another is a filamentous fungus including *Aspergillus, Trichoderma, Mucor* and *Fusarium*. In yet a further embodiment, the *Aspergillus* species includes, but is not limited to, *A. niger, A. nidulans, A. oryzae* and *A. fumigatus*.

The present invention also provides a method for producing a desired protein in a fungus comprising the step of culturing a recombinant fungus comprising a polynucleotide encoding the desired protein under conditions suitable for the production of said desired protein, said recombinant fungus further comprising a polynucleotide encoding a protein associated with hyphal growth in said fungus said protein associated with hyphal growth

having at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the amino acid sequence as disclosed in SEQ ID NO:2. In one embodiment, the polynucleotide encoding a protein associated with hyphal growth is homologous to said fungus and is present in amounts greater than found in the naturally occurring fungus. In another embodiment, the polynucleotide encoding a protein associated with hyphal growth is heterologus to said fungus and has been recombinantly introduced into said fungus. The method may further comprise the step of recovering said desired protein.

In another aspect of the present invention, it may be desirable to down regulate expression of the protein associated with hyphal growth in order to reduce culture viscosity. Accordingly, the present invention provides a method for producing a desired protein in a fungus comprising the step of culturing a recombinant fungus comprising a polynucleotide encoding the desired protein under conditions suitable for the production of said desired protein, said recombinant fungus comprising a mutation in an endogenous nucleic acid encoding a protein associated with hyphal growth said mutation resulting in the inhibition of the production by said fungus of the protein associated with hyphal growth.

In one embodiment, the polynucleotide encoding a protein associated with hyphal growth in said fungus comprises a replicating plasmid. In another embodiment, the polynucleotide encoding a protein associated with hyphal growth in said fungus is integrated into the fungal genome. In yet a further embodiment, the protein associated with hyphal growth has the amino acid sequence as shown in SEQ ID NO:2.

In yet a further embodiment of the present invention, the polynucleotide encoding a protein associated with hyphal growth has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the polynucleotide having the sequence as shown in SEQ ID NO: 1, or is capable of hybridizing to the polynucleotide having the sequence as shown in SEQ ID NO:1 under conditions of intermediate to high stringency, or is complementary to the polynucleotide having the sequence as shown in SEQ ID NO:1. In another embodiment, the polynucletolde has the nucleic acid sequence as shown in SEQ ID NO: 1.

The present invention also provides a method for producing a recombinant fungus comprising a polynucleotide encoding a protein associated with hyphal growth comprising the steps of obtaining a polynucleotide encoding said protein associated with hyphal growth; introducing said polynucleotide into said host cell; and growing said host cell under conditions suitable for the production of said protein associated with hyphal growth. In one embodiment of this method, the host cell is a fungus. In another embodiment, the filamentous fungus includes *Aspergillus*, *Trichoderma*, *Mucor* and *Fusarium* species. In yet

another embodiment, the *Aspergillus* species includes *A. niger*, *A. nidulans*, *A. oryzae* and *A. fumigatus*. In one embodiment, the polynucleotide has at least 60% identity to the nucleic acid having the sequence as shown in SEQ ID NO:1 or is capable of hybridizing to the polynucleotide having the sequence as shown in SEQ ID NO:1 under conditions of intermediate to high stringency, or is complementary to the polynucleotide having the sequence as shown in SEQ ID NO:1. In another embodiment, the polynucleotide has the sequence as shown in SEQ ID NO:1.

The present invention also relates to methods for screening for mutants exhibiting a hyper branching phenotype and which are capable of secreting heterologous protein. Accordingly, the present invention provides a method for the identification of hyper-branching mutants which comprise the steps of obtaining fungal mutants, subjecting said mutants to selection under desired conditions, and identifying mutants having the desired phenotypes. In one embodiment, the identification comprises selecting for hyphal growth. In yet another embodiment, identification comprises selecting for mutants capable of secreting protein. In another embodiment, the selection comprises growth and/or secretion of heterologous proteins at a restricted temperature.

Brief Description of the Drawings

Figures 1A-1D illustrates the nucleic acid (SEQ ID NO:1, *hbrA*) and amino acid (SEQ ID NO:2) sequence for HbrA.

Figures 2A-2B illustrates an amino acid alignment of the amino acid sequence for *hbrA*; A. fumigatus (afvac); rat (ratvac); yeast slp gene (slp1_yeast); C.elegans (slp1_ceel).

Figure 3 illustrates amylase secretion by hbr/creA mutants.

Detailed Description of the Invention

Definitions

As used herein, the phrase "protein associated with hyphal growth" refers to a protein which is capable of modulating hyphal growth in fungus. Illustrative of such proteins are the proteins HbrA 1-9 disclosed herein in the Examples. The term "HbrA" refers to the amino acid sequence as shown in SEQ ID NO:2. The present invention encompasses proteins associated with hyphal growth in fungus having at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the amino acid sequence as disclosed in SEQ ID NO:2. Percent identity at the nucleic acid level is determined using the FastA program and percent identity at the amino acid level is determined using the TFastA both of which use the method of Pearson and Lipman (PNAS USA, 1988, 85:2444-2448). The present invention also encompasses mutants, variants and derivatives of HbrA as long as the mutant, variant or derivative is capable of modulating hyphal growth in fungus.

As used herein, "nucleic acid" refers to a nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. As used herein "amino acid" refers to peptide or protein sequences or portions thereof.

The terms "isolated" or "purified" as used herein refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

As used herein, the term "heterologous" when refering to a protein associated with hyphal growth refers to a protein that does not naturally occur in a fungal cell. The term "homologous" when refering to a protein associated with hyphal growth refers to a protein native or naturally occurring in the fungus. The invention includes fungal host cells producing the homologous protein associated with hyphal growth at higher copy number than found in the naturally occurring fungal host and produced at a higher copy level via recombinant DNA technology.

As used herein, the term "overexpressing" when referring to the production of a protein in a host cell means that the protein is produced in greater amounts than its production in its naturally occurring environment.

Description of the Preferred Embodiments

The present invention relates to the identification of HbrA in *A. nidulans*. The mutation of HbrA, referred to herein as HbrA2, was assigned to chromosome VII by parasexual analysis (*Aspergillus*: 50 Years On (1994) vol 20, ed S.D. Martinelli & J.R. Kinghorn pp. 41-43). At 37°C, mutant *hbrA2*, unlike wild-type *A. nidulans*, fails to secrete recombinantly expressed *M. meihei* protease. The translated sequence of the *hbrA2* gene shows significant identity with the yeast SLP/VPS33 Sec1 gene product. Available evidence indicates that SLP/VPS33 Sec1 encodes a protein essential for vacuolar protein sorting. SLP1 mutants fail to direct proteins to the vacuoles, and they are sent along a default pathway to the cytoplasmic membrane. The exact nature and function of the SLP1/VPS33 Sec1 protein is unknown, but it is a member of the SEC1 family, and may be a membrane associated protein involved in directing vesicles to vacuoles. Deletion of VPS33 in yeast in not lethal, but leads to slow growth, temperature sensitivity, and loss of vacuoles as revealed by staining light and electron microscopy. Fluorescence microscopy has shown that like SLP1/VSP33 mutants in yeast, HbrA2 is defective in vacuole assembly at the non-permissive temperature.

The mutation HbrA2 does not appear to affect growth of *A. nidulans* at 26°C, but results in a hyperbranching, restricted growth phenotype at 42°C. The hyperbranching

phenotype shows extensive branching in the apical compartment, unlike the wild-type *A.nidulans*. The mutant grows slowly at the non-permissive temperature giving rise to highly compact colonies on agar media. *Mucor meihei* protease was transformed into wild-type *A.nidulans* and crossed into the *hbrA*2 mutant. The *hbrA*2 mutant comprising the heterologous nucleic acid encoding the *M.meihei* protease was able to secrete the protease at 26°C. The *hbrA*2 mutant was unable to secrete the protease at 37°C but was able to secrete the endogenous alpha amylase at temperatures greater than 37°C.

In view of the observation that *hbrA* mutants are incapable of producing foreign protein, it appears that genetically engineering fungal hosts to modulate the expression of proteins associated with hyphal growth, in particular, mutants HbrA1-9, would provide a means for enhancing the production of proteins or chemicals in the fungal host. In one aspect of the present invention, it would be desirable to increase expression of proteins associated with hyphal growth. In another aspect of the present invention, it would be desirable to decrease or eliminate expression of proteins associated with hyphal growth by means known to the skilled artisan.

I. HbrA amino acid and *hbrA* nucleic acid sequences

The present invention provides the amino acid (SEQ ID NO:2) HbrA and nucleic acid (SEQ ID NO:1) sequence for *hbrA*. The present invention encompasses amino acid variants having at least 70% identity to the amino acid having the sequence as shown in SEQ ID NO:2 as long as the variant is capable of modulating hyphal growth. Percent identity at the nucleic acid level is determined using the FastA program and percent identity at the amino acid level is determined using the TFastA both of which use the method of Pearson and Lipman (PNAS USA, 1988, 85:2444-2448). Alternatively, identity is determined by MegAlign Program from DNAstar (DNASTAR, Inc. Maidson, WI 53715) by Jotun Hein Method (1990, Method in Enzymology, 183: 626-645) with a gap penalty = 11, a gap length penalty = 3 and Pairwise Alignment Parameters Ktuple = 2. As the skilled artisan will readily recognize, a variety of polynucleotides can encode HbrA. The present invention encompasses all such polynucleotides. *HbrA*, and other polynucleotides encoding proteins associated with hyphal growth, may be obtained by standard procedures known in the art from, for example, cloned DNA (*e.g.*, a DNA "library"), genomic DNA libraries, by chemical synthesis once identified, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from a desired cell. (*See*, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Nucleic acid

sequences derived from genomic DNA may contain regulatory regions in addition to coding regions. Whatever the source, the isolated polynucleotide encoding the protein associated with hyphal growth can be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the gene may be accomplished in a number of ways. For example, a polynucleotide encoding a protein associated with hyphal growth or its specific RNA, or a fragment thereof, such as a probe or primer, may be isolated and labeled and then used in hybridization assays to detect related genes. (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci. USA 72:3961). Those DNA fragments sharing substantial sequence similarity to the probe will hybridize under stringent conditions.

Also included within the scope of the present invention are fungal microorganism polynucleotide sequences that are capable of hybridizing to the nucleotide sequence of SEQ ID NO:1 under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA) incorporated herein by reference, and confer a defined "stringency" as explained below.

"Maximum stringency" typically occurs at about Tm-5°C (5°C below the Tm of the probe); "high stringency" at about 5°C to 10°C below Tm; "intermediate stringency" at about 10°C to 20°C below Tm; and "low stringency" at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY).

The process of amplification as carried out in polymerase chain reaction (PCR) technologies is described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY). A nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides from SEQ ID NO:1 preferably about 12 to 30 nucleotides, and more preferably about 20-25 nucleotides can be used as a probe or PCR primer.

Expression Systems

The present invention provides host cells, expression methods and systems for the production of desired proteins in host fungus. Once nucleic acid encoding a protein associated with hyphal growth is obtained, recombinant host cells containing the nucleic acid may be constructed using techniques well known in the art. Molecular biology techniques are disclosed in Sambrook et al., Molecular Biology Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). Nucleic acid encoding proteins associated with hyphal growth and having at least 60% identity to *hbrA* is obtained and transformed into a host cell using appropriate vectors. A variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression in fungus are known by those of skill in the art.

Typically, the vector or cassette contains sequences directing transcription and translation of the nucleic acid, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. These control regions may be derived from genes homologous or heterologous to the host as long as the control region selected is able to function in the host cell.

Initiation control regions or promoters, which are useful to drive expression of the protein associated with hyphal growth in a host cell are known to those skilled in the art. Virtually any promoter capable of driving these proteins is suitable for the present invention. Nucleic acid encoding the protein is linked operably through initiation codons to selected expression control regions for effective expression of the protein. Once suitable cassettes are constructed they are used to transform the host cell.

General transformation procedures are taught in Current Protocols In Molecular Biology (vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, Chapter 9) and include calcium phosphate methods, transformation using PEG and electroporation. For *Aspergillus* and *Trichoderma*, PEG and Calcium mediated protoplast transformation can be used (Finkelstein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi.

Technology and Products (eds by Finkelstein & Bill) 113-156. Electroporation of protoplast is disclosed in Finkelestein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi. Technology and Products (eds by Finkelstein & Bill) 113-156. Microprojection bombardment on conidia is described in Fungaro et al. (1995) Transformation of *Aspergillus* nidulans by microprojection bombardment on intact conidia. FEMS Microbiology Letters 125 293-298. Agrobacterium mediated transformation is disclosed in Groot et al. (1998) Agrobacterium tumefaciens-mediated transformation of filamentous fungi. Nature Biotechnology 16 839-842.

Host cells which comprise the sequence for *hbrA* and express the protein may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein. For production of a desired protein in a fungal host cell, an expression vector comprising at least one copy of nucleic acid encoding a desired protein is transformed into the recombinant host cell comprising nucleic acid encoding a protein associated with hyphal growth and cultured under conditions suitable for expression of the protein. Examples of desired proteins include enzymes such as hydrolases including proteases, cellulases, amylases, carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases and phophatases along with proteins of therapeutic value. Alternatively, it may be advantageous to down-regulate or mutate proteins associated with hyphal growth in order to reduce the viscosity in the fermentor.

III Vector Sequences

Expression vectors used in expressing the *hprA* in fungal cells or the desired protein in fungal cells comprise at least one promoter associated with the protein which promoter is functional in the host cell. In one embodiment of the present invention, the promoter is the wild-type promoter for the protein and in another embodiment of the present invention, the promoter is heterologous to the protein, but is still functional in the fungal host cell. In one preferred embodiment of the present invention, nucleic acid encoding the protein is stably integrated into the microorganism genome.

In a preferred embodiment, the expression vector contains a multiple cloning site cassette which preferably comprises at least one restriction endonuclease site unique to the vector, to facilitate ease of nucleic acid manipulation. In a preferred embodiment, the vector also comprises one or more selectable markers. As used herein, the term

selectable marker refers to a gene capable of expression in the host which allows for ease of selection of those hosts containing the vector.

IV. Assay of the activity of proteins associated with fungal growth

The results shown in Examples I and II illustrate the use of a temperature based screen to identify mutants which effect fungal branching. The unexpected advantage of using such a temperature based screen is the ability to identify HbrA mutants or mutants of proteins associated with hyphal growth having a differential effect on the export of native or endogenous genes vs the export of recombinantly introduced heterologous protein. This type of screening method facilitates the isolation of strains which are capable of increased secretion of heterologous protein. Therefore, the present invention also provides a method for the identification of hyper-branching mutants which enhance protein secretion comprising the steps of obtaining fungal mutants, subjecting said mutants to selection under desired conditions, and identifying the desired mutants. In one embodiment, the identification comprises selecting for hyphal growth. In another embodiment, the selection comprises growth and/or secretion of heterologous proteins at a restricted temperature.

Examples

Example I

This example illustrates the isolation of the *hbrA* gene. In order to isolate the *hbrA* gene, DNA was prepared from pooled cosmids of the chromosome-sorted cosmid library of wild-type DNA from *A. nidulans* obtained from FGSC (Funal Genetic Stock Center, Department of Microbiology University of Kansas Medical Center, Kansas City, KS 66160). 5 pools of 20 cosmids each were used in transformation experiments. In order to assess transformation efficiency, an *hbrA*2, argB double mutant was used as a recipient for cotransformation using a mixture of cosmid DNA and transforming vector Arp, which carries the argB gene and a replicating sequence. After transformation, protoplasts were regenerated and selected on medium lacking arginine at 42°C. One of the cosmid pools gave rise to a few strongly growing, normally conidiating colonies in a background of Arg+ Hbr- transformants. The pool was subdivided into 4 pools of 5 cosmids, and transformation repeated. A single cosmid was isolated which was able to complement the *hbrA*2 mutation, restoring wild-type growth. Sub-cloning of the cosmid led to identification of an EcoRI fragment carrying the transforming sequence. The EcoRI/BamHI fragments failed to complement the mutation suggesting that the BamHI site lies within the *hbrA* gene. The fragment was isolated and subjected to nucleic acid sequencing. The nucleic acid and amino acid sequence for the *hbrA* gene is shown in Figures 1A-1D. Table I shows

protease activity for Hbr2, as well as other identified hyper-branching mutants at the permissive and non-permissive temperatures.

Table I

Strain	Mean Protease Activity (units/gram of biomass) at 26C		Mean Protease Activity (units/gram of biomass) at (37C)	
	48 hrs	72 hrs	48hrs	72 hrs
Wild-type	963+/- 57	703+/-12	380+/-44	339+/- 40
HbrA2	857+/-18	1237+/-155	0+/- 0	0+/-0
Hbr3	689+/-76	1194+/-234	0+/-0	0+/-0
Hbr6	0+/-0	1892+/-122	0+/-0	0+/-0
Hbr8	0+/-0	2165+/-156	0+/-0	487+/-10

These findings indicate that a previously uncharacterized filamentous fungal gene *hbrA* plays a role in heterologous protein export.

Example 2

This Example describes the characterization of hyperbranching mutants of *A. nidulans*. Below is Table II which shows the chromosomal location of the hbr mutants.

hbr Mutant	Chromosomal location
hbr1	I
hbrA2	VII
hbr3	I
hbr4	III
hbr5	VIII
hbr6	III
hbr7	III
hbr8	I
hbr9	III

All mutations were recessive and unlinked to each other and represent previously uncharacterized mutations which effect fungal hyperbranching and protein secretion. The ability of *hbrA2* mutant to secrete the endogenous protein alpha amylase at 37°C was examined by growing the *hbrA2*:creA- double mutant on petri dishes with starch as the sole carbon source (the CreA gene is a negatively acting regulator of carbon catabolism repression. Mutations of CreA (CreA-) causes carbon catabolism derepression of enzymes such as alpha amylase). The *hbrA2*:creA- double mutant like the *hbrA+*:creA- was shown to be capable of secreting the endogenous protein alpha amylase, see Figure 3.

These results indicate the *hbrA* gene unexpectantly plays a role in heterologous protein secretion.

The hbr3 mutant, like the *hbrA*2 mutant, produces slightly higher *M. meihei* protease than the wild-type at 26°C. At 37°C, the *hbr3* mutant like the *hbrA*2 mutant does not produce the *M. meihei* protease. The *hbrA*2 mutation is located on chromosome VII, the *hbr3* mutation is located on chromosome I. These results indicate that the *hbr3* gene product also plays a role in heterologous protein export. Therefore, modulation of the expression of the wild-type *hbr3* gene product would appear to be advantageous in increasing heterologous protein export.

The *hbr6* and *hbr8* mutations which are located on chromosomes III and I respectively, produce significantly higher levels of *M. meihei* protease than the wild-type at 26°C and would appear to increase the secretion of heterologous protein in a filamentous fungus grown in the temperature range around 26°C. Therefore, modulation of expression of the wildtype *hbr6* and *hbr8* gene products would also appear to have utility in increasing heterolgous protein export. Mutant versions of the *hbr6* and *hbr8* genes have no or significantly less *M. meihei* secretion than the wild-type as shown by Table III.

Table III

Strain	Mean Protease Activity (units/gram of biomass) at 26C		Mean Protease Activity (units/gram of biomass) at 37C	
	48 hrs	72 hrs	48hrs	72 hrs
Wild-type	963+/- 57	703+/-12	380+/-44	339+/- 40
hbr5	46+/-60	1152+/-133	533+/-53	1648+/-797
hbr7	0+/-0	1098+/-53	580+/-60	1581+/-660
hbr4	844+/-114	1688+/-67	343+/-26	260+/-15
hbr9	0+/-0	268+/-16	0+/-0	1562+/641

Table II illustrates that *M. meihei* protease secretion in the *hbr5* and *hbr7* mutants yields slightly more protease at 26°C after 72 hours compared to the wild-type, and significantly more protease at 72 hours at 37°C.

The *hbr4* mutant produced significantly more M. meihei protease than the wild-type after 72hours at 26°C but significantly less protease after 72 hours at 37°C. However, the *hbr4*:creA- double mutant produced significantly higher levels of alpha amylase/unit area fungal colony that the wild-type strain containing only the creA- mutation. These results indicate a significant role for the *hbr4* gene product not only in terms of fungal morphology

increasing native protein secretion but also a role for this gene product in heterologous protein export.

The *hbr9* mutation exhibited poor expression of *M. meihei* protease at 26°C, but significantly higher levels of *M. meihei* protease and alpha amylase/ unit area fungal colony than the wild-type.

Claims

We claim:

1. An isolated protein associated with hyphal growth in fungi having at least 70% identity to the amino acid sequence as disclosed in SEQ ID NO:2.

2. The protein of Claim 1 having the amino acid sequence as disclosed in SEQ ID NO:2.

3. An isolated polynucleotide encoding the amino acid having the sequence as shown in SEQ ID NO:2.

4. The isolated polynucleotide of Claim 3 having at least 60% identity to the polynucleotide have the sequence as shown in SEQ ID NO: 1, or is capable of hybridizing to the polynucleotide having the sequence as shown in SEQ ID NO:1 under conditions of intermediate to high stringency, or is complementary to the polynucleotide having the sequence as shown in SEQ ID NO:1.

5. The isolated polynucleotide of Claim 4 having the nucleic acid sequence as disclosed in SEQ ID NO:1.

6. An expression vector comprising the polynucleotide of Claim 3.

7. A host cell comprising the expression vector of Claim 6.

8. The host cell of Claim 7 that is a filamentous fungus.

9. The host cell of Claim 8 wherein said filamentous fungus includes *Aspergillus*, *Trichoderma*, *Mucor* and *Fusarium*.

10. A method for producing a desired protein in a fungus comprising the step of, culturing a recombinant fungus comprising a polynucleotide encoding the desired protein under conditions suitable for the production of said desired protein, said recombinant fungus further comprising a polynucleotide encoding a protein associated with hyphal growth in said fungus said protein having at least 70% identity to the amino acid sequence as disclosed in SEQ ID NO:2.

11. The method of Claim 10 further comprising the step of recovering said desired protein.

12. The method of Claim 10 wherein said polynucleotide encoding a protein associated with hyphal growth is homologous to said fungus said polypeptide being present in copy number greater than found in the naturally occurring fungus.

13. The method of Claim 10 wherein the polynucleotide encoding a protein associated with hyphal growth is heterologus to said fungus and has been recombinantly introduced into said fungus.

14. The method of Claim 10 wherein said polynucleotide encoding a protein associated with hyphal growth in said fungus comprises a replicating plasmid.

15. The method of Claim 10 wherein said polynucleotide encoding a protein associated with hyphal growth in said fungus is integrated into the fungal genome.

16. The method of Claim 10 wherein said protein associated with hyphal growth has the amino acid sequence as shown in SEQ ID NO:2.

17. The method of Claim 10 wherein said polynucleotide encoding a proteln associated with hyphal growth has 60% identity to the polynucleotide having the sequence as shown in SEQ ID NO:1, or is capable of hybridizing to the polynucleotide having the sequence as shown in SEQ ID NO:1 under conditions of intermediate to high stringency, or is complementary to the polynucleotide having the sequence as shown in SEQ ID NO:1.

18. The method of Claim 15 wherein said polynucletoide has the nucleic acid sequence as shown In SEQ ID NO: 1.

19. The method of Claim 10 wherein said fungus is a filamentous fungus.

20. The method of Claim 19 wherein said filamentous fungus includes *Aspergillus*, *Trichoderma*, Mucor and *Fusarium* species.

21. The method of Claim 20 wherein the *Aspergillus* includes *A. niger*, *A. nidulans*, *A. oryzai* and *A. fumigatus*.

22. A method for producing a recombinant fungus comprising a polynucleotide encoding a protein associated with hyphal growth comprising the steps of:

(a) obtaining a polynucleotide encoding said protein associated with;

(b) introducing said polynucleotide into said host cell; and

(c) growing said host cell under conditions suitable for the production of said protein associated with hyphal growth.

23. The method of Claim 22 wherein said host cell includes filamentous fungus.

24. The method of Claim 23 wherein said filamentous fungus includes *Aspergillus*, *Trichoderma*, *Mucor*, and *Fusarium*.

25. The method of Claim 24 wherein said *Aspergillus* species includes *A. niger*, *A. nidulans*, *A. oryzae* and *A. fumigatus*.

26. The method of Claim 22 wherein said polynucleotide has at least 60% identity to the nucleic acid having the sequence as shown in SEQ ID NO:1.

27. A method of identifying hyper-branching fungal mutants comprising the steps of obtaining fungal mutants, subjecting said mutants to selection under desired conditions, and identifying the desired mutants.

28. The method of Claim 27 wherein said identifying comprises selecting for hyphal growth.

29. The method of Claim 27 wherein the selection comprises growth and secretion of heterologous proteins at a restricted temperature.

30. A method for producing a desired protein in a fungus comprising the step of culturing a recombinant fungus comprising a polynucleotide encoding the desired protein under conditions suitable for the production of said desired protein, said recombinant fungus comprising a mutation in an endogenous nucleic acid encoding a protein associated with hyphal growth said mutation resulting in the inhibition of the production by said fungus of the protein associated with hyphal growth.

31. The method of Claim 30 wherein said protein associated with hyphal growth has at least 70% identity to the amino acid sequence as disclosed in SEQ ID NO:2.

```
        10                  30                  50
        .                   .                   .
GATCACCAGGAATTGCGTTGCCTGATGCATGGTTGGGAGGGCCGCCGAGGTCCACGCCAG

        70                  90                  110
        .                   .                   .
GTGGTGGGGGTGCTATACCGTGCTGCGCTTTTGCCCTCGTGTAAGGGTCAGCAGGAATCG

        130                 150                 170
        .                   .                   .
GTTTCGCGTAAGGATTCGCTTCGGCAGGAGGGCTCTTGTTCTTCGACCTCGATCCAAAGA

        190                 210                 230
        .                   .                   .
GGGCGCGGCGGTTGGAGGAATCGTCGTCGCCGGCGTCTGACGACTTTTTGAGGCCGAATC

        250                 270                 290
        .                   .                   .
GCTTCATAGCGTATTTTAGCTAGAATACTTCGCCGAAAcCAGCGTAGGAATAtTAGAGTG

        310                 330                 350
        .                   .                   .
AAAATAATAAATTGAGAGGCTATTTATGATTGACTGAGAATTGAAGAGAGGGGAAGGGAA

        370                 390                 410
        .                   .                   .
GGAGGGAGGGGAGCGAAGATGTTAAGTGTCAGGGGAGCAGCAGCGGCAAAAGTGTCAAGA

        430                 450                 470
        .                   .                   .
CGCTCCTGAGACTCAAAGGCAGCTATGTAATCATGATACACATAGTTGTGCTGCAATTCT

        490                 510                 530
        .                   .                   .
GGCTATCAGTGAGTATTTTACCGTATGATTACTCACCAATTCGACTCCACTAAGCCGAAA

        550                 570                 590
        .                   .                   .
GAAGCTAGCGGGGATGGCTGGACCCTTCTAAGCCTCAACTGAGGGCGGTGCCGCAGTCAA

        610                 630                 650
        .                   .                   .
ACGTCAACTGCTCCCACCCCATGCTTCGTATAAGGTAGCCATGGCACCATTCCCTGGGTC

        670                 690                 710
        .                   .                   .
TGATGCCGACAATATCAAGGACAAGGCCCGTAAAGGCTTGCTGAATCTTCTCGAAGGCGT
```

FIG._1A

```
       730                 750                 770
        .                   .                   .                   .
GAGTAAGGCTCCTAGTTGGCACTGTTTCTGGTTCTAGCCTGATTCATTACCTCGATCTAG

       790                 810                 830
        .                   .                   .                   .
GTCCGTGGGAAGAAGAACCTGGTGATTAGCCAGGGGCTTGCTGGGCCCGTCGGGCTTTTT

       850                 870                 890
        .                   .                   .                   .
GTCAAGTTTTCGCAGCTTCAGGAGTATGGCGTAGACCGGGTATTCTTGCTTGAAAATGGA

       910                 930                 950
        .                   .                   .                   .
AATGTCGACTCTTCTCAGCGCAATGTGGTATTTCTAGCGTACGCCGAAAAGATCCGCCAG

       970                 990                1010
        .                   .                   .                   .
GTGCGGGCAGTGGCAGGTATGTCATGATCTTTATCCACCTTTGATTTACATACCCAAATG

      1030                1050                1070
        .                   .                   .                   .
ACTGTAAATGCGAAGGCTCCTTGCTATCGCGCTTGCTGGGAGCATTAAAGTTACGCAGAC

      1090                1110                1130
        .                   .                   .                   .
TTCTTCTCCACTCTGCGTAATCAGTCAAGCTCCCTATATTGAAACTTCGTTTAGCAGCTT

      1150                1170                1190
        .                   .                   .                   .
ATCCCTAAGGCTTTCTTTCTCTGCCTCGTATGACTGAATGCCATCAGAATAAGCTGACAA

      1210                1230                1250
        .                   .                   .                   .
GTTTTACAGAGCAGATCCAAAGGCTTCAACGCAACAGCAGTATAGACCATGAATTTTCCA
                                                  H   E   F   S   I

      1270                1290                1310
        .                   .                   .                   .
TCTTTTGGGTTCCAAGACGGACCCTCGTAAGCAATAACATCCTAGAGAGCGCAGGCATCA
  F   W   V   P   R   R   T   L   V   S   N   N   I   L   E   S   A   G   I   I

      1330                1350                1370
        .                   .                   .                   .
TTGGAGATGTGAGCATCGCTGAGCTGCCTCTTTACTTTTTTCCTCTAGAGCAGGACGTTC
  G   D   V   S   I   A   E   L   P   L   Y   F   F   P   L   E   Q   D   V   L
```

FIG._1B

```
      1390                1410                1430
       .         .         .         .         .         .
TTTCTTTGGAACTGGATGACTCTTTTGCGGACTTGTACCTGGTGAGATCTTTCTCCTGGA
  S   L   E   L   D   D   S   F   A   D   L   Y   L

      1450                1470                1490
       .         .         .         .         .         .
GATAGTGATCAGTGCTGATTCATTTTGTAGCACAAGGAtccTGGGTGCATCTTCCATTCC
                                H   K   D   P   G   C   I   F   H   S

      1510                1530                1550
       .         .         .         .         .         .
GCAAAGGCTCTTATGgctATTCAACAGAGACATGGCTATTTTCCTCGGATAGTAGGCAAA
A   K   A   L   M   A   I   Q   Q   R   H   G   Y   F   P   R   I   V   G   K

      1570                1590                1610
       .         .         .         .         .         .
GGCGATCATGCTCGACGACTCGCTGACCTCCTGCTGCGGATGAGGAAGGAGATTGACGCA
G   D   H   A   R   R   L   A   D   L   L   L   R   M   R   K   E   I   D   A

      1630                1650                1670
       .         .         .         .         .         .
GAGGAAAGCTCAGGACTGACAGGACTGTCTTTCCGGGGACTTTTACCCAGCTCAAGCATT
E   E   S   S   G   L   T   G   L   S   F   R   G   L   L   P   S   S   S   I

      1690                1710                1730
       .         .         .         .         .         .
GAGAGTTTGATCATCATTGACCGAGAGGTGGACTTCGGCACCCCTCTGCTTACACAGCTA
E   S   L   I   I   I   D   R   E   V   D   F   G   T   P   L   L   T   Q   L

      1750                1770                1790
       .         .         .         .         .         .
ACGTATGAGGGTCTCATCGATGAGTTGGTAGGAATCAAGCACAACCAAGCGGACATTGAT
T   Y   E   G   L   I   D   E   L   V   G   I   K   H   N   Q   A   D   I   D

      1810                1830                1850
       .         .         .         .         .         .
ACGACAATTGCAGGGGCCAGCTCAACTCCCCAGGCCCAGGAGTCTTCCAAAGCATCTCAA
T   T   I   A   G   A   S   S   T   P   Q   A   Q   E   S   S   K   A   S   Q

      1870                1890                1910
       .         .         .         .         .         .
CAGGCTAAGCAAGGTCAAAAGCGGAAGATTCAGTTGGATTCGTCTGACCAACTGTTCAGT
Q   A   K   Q   G   Q   K   R   K   I   Q   L   D   S   S   D   Q   L   F   S
```

FIG._1C

```
      1930                1950                1970
       .                   .                   .                   .
CAACTCCGTGACGCGAATTTTGCTATAGTCGGCGATATCCTGAATAAGGTAGCACGTCGA
Q  L  R  D  A  N  F  A  I  V  G  D  I  L  N  K  V  A  R  R

      1990                2010                2030
       .                   .                   .                   .
TTAGAAACAGATTATGAGAGCCGTCATACAGCAAAAACGACAACTGAACTTCGCGAGTTT
L  E  T  D  Y  E  S  R  H  T  A  K  T  T  T  E  L  R  E  F

      2050                2070                2090
       .                   .                   .                   .
GTGAATAAACTACCATCATATCAACTCGAACATCAAAGCTTGAGAGTTCACACCAACCTC
V  N  K  L  P  S  Y  Q  L  E  H  Q  S  L  R  V  H  T  N  L

      2110                2130                2150
       .                   .                   .                   .
GCTGAGGAAATCATGAAAAACACGCGCTCAGACACTTTCCGCAAGATCCTCGAAGTGCAA
A  E  E  I  M  K  N  T  R  S  D  T  F  R  K  I  L  E  V  Q

      2170                2190                2210
       .                   .                   .                   .
CAGAACGACGCTGCAGGCGCCGACCCAACTTACCAACATCCTCTCATTGAGGAACTCATC
Q  N  D  A  A  G  A  D  P  T  Y  Q  H  P  L  I  E  E  L  I

      2230                2250                2270
       .                   .                   .                   .
GCCCGGGATATTCCACTGAAGACAATCCTCCGTTTGCTTTGTCTCGAATCATGCATGTCC
A  R  D  I  P  L  K  T  I  L  R  L  L  C  L  E  S  C  M  S

      2290                2310                2330
       .                   .                   .                   .
GGTGGCCTACGGCCTAAAGACCTCGAGAGTTTTAAACGCCAAGTCGTCCACGCATACGGG
G  G  L  R  P  K  D  L  E  S  F  K  R  Q  V  V  H  A  Y  G

      2350                2370                2390
       .                   .                   .                   .
CACCAACACCTGCTAACATTCAGTGCTTTGGAGAAGATGGAGCTTCTCCAGCCCCGGTCG
H  Q  H  L  L  T  F  S  A  L  E  K  M  E  L  L  Q  P  R  S

      2410                2430                2450
       .                   .                   .                   .
TCTGCAACCACAATGCTAATTCCCGGCACGGGCACCCAAACGGGATCGAAAACAAACTAC
S  A  T  T  M  L  I  P  G  T  G  T  Q  T  G  S  K  T  N  Y
```

FIG._1D

```
      2470                2490                2510
      .                   .                   .                   .
GCCTACTTTCGCAAAAATCTTCGCCTGGTCGTCGAAGAAGTTAGCGAGAAGGAACCTGAA
A  Y  F  R  K  N  L  R  L  V  V  E  E  V  S  E  K  E  P  E

      2530                2550                2570
      .                   .                   .                   .
GATATCGCTTATGTCTACAGCGGTTTCGCCCCTCTCAGCATTCGCCTTGTGCAGTGCGTC
D  I  A  Y  V  Y  S  G  F  A  P  L  S  I  R  L  V  Q  C  V

      2590                2610                2630
      .                   .                   .                   .
TTGCAGAAATCATACGTCATGTCGCTTATGAAAGGTGGCCCGGCTGCGCACGCGAATACC
L  Q  K  S  Y  V  M  S  L  M  K  G  G  P  A  A  H  A  N  T

      2650                2670                2690
      .                   .                   .                   .
GCATCCCCAGGCTGGCTTGGATATGAAGATGTGGTGAAGAGTGCGCGTGGATCGACGTTC
A  S  P  G  W  L  G  Y  E  D  V  V  K  S  A  R  G  S  T  F

      2710                2730                2750
      .                   .                   .                   .
AGTATTGTCCAAAAGGGCGACGATAAAGCGGTTCGTGCGCGGCAGACACTGAGTGGTAAC
S  I  V  Q  K  G  D  D  K  A  V  R  A  R  Q  T  L  S  G  N

      2770                2790                2810
      .                   .                   .                   .
AATGCGGCTAAGACCGTGTATGTGTTCTTCCTCGGAGGGATCACATTTACGGAAATCGCG
N  A  A  K  T  V  Y  V  F  F  L  G  G  I  T  F  T  E  I  A

      2830                2850                2870
      .                   .                   .                   .
GCATTGCGGTTCATTGCGGCACAGGAGGCGCCGAGGCGGAACATTGTGATTTGTACTACG
A  L  R  F  I  A  A  Q  E  A  P  R  R  N  I  V  I  C  T  T

      2890                2910                2930
      .                   .                   .                   .
GGAATCATTAATGGAGATCGGATGATGGATGCTGCGCTTGAGAAGGGGGGGTTTGCCTTG
G  I  I  N  G  D  R  M  M  D  A  A  L  E  K  G  G  F  A  L

      2950                2970                2990
      .                   .                   .                   .
ACTGAGTCTTGACCTCGTAGAGCGTACAGTTAATGTCATAGGAACTATACCGCTATCCAT
T  E  S
```

FIG._1E

```
hbra       .......... .......... .......... .......... .......... .......... 0
afvac      .......... .......... .......... .......... .......... .......... 0
ratvac     MAAHLSYGRV NLNVLREAVR RELREFLDKC AGSKAIVWDE YLTGPFGLIA QYSLLKEHEV 60
slp1_yeast ....MNRFWN TKKFSLTNAD GLCATLNEIS QNDEVLVVQP SVLPVLNSLL TFQDLTQSTP 56
slp1_caeel .......... .......... .......MAA NEDRDDAAAI LNWEGTSEIK SANEYSRNLL 33

hbra       .......... .......... .......... .......... .......... ..HEFSIFWV 8
afvac      .......... .......... .......... .......... .......... ..HEFSIFWL 8
ratvac     EKMFTLKGSR LPAADVKNII FLVRPRLELM D......MIA ENVLSEDRRG PTRDFHILFV 114
slp1_yeast VRKITLLDDQ LSDDLPSALG SVPQMDLIFL IDVRTSLRLP PQLLDAAQKH NLSSLHIIYC 116
slp1_caeel FSVLDSLDGN KTIVWDRDRS VMHRVNLFAG ASVLAAHGVV ANHSIETKKS ASTPHVVFFL 93

hbra       PRRTLVSNNI LES.......  .......... AGIIGD.VSI AE.....LPL YFFPL...EQ 42
afvac      PRRTFVSNKI LED.......  .......... AGIIGD.VNI FE.....FPL YFVPL...EQ 42
ratvac     PRRSLLCEQR LKD.......  .......... LGVLGSFIYR EE.....YSL DLIPF...DG 149
slp1_yeast RWKPSFQNTL EDTEQWQKDG FDLNSKKTHF PNVIESQLKE LSNEYTLYPW DLLPFPQIDE 176
slp1_caeel A.PTMVSLDL LCD.......  .........YI DNVRNDSYWE RLESVKEIPL CWLP...RDG 134

hbra       DVLSLELDDS FADLYLHKDP GCIFHSAKAL MAIQQR....  .......HGY FPRIVGKGDH 91
afvac      DVLSLELDDS FGDLYLHKDP GCIFLAAKAL MDIQQR....  .......HGY FPRIIGKGDH 91
ratvac     DLLSMESESA FKECYLEGDQ TSLYHAAKGL MTLQAL....  .......YGT IPQIFGHGEC 198
slp1_yeast NVLLTHSLYN MENVNMYYPN LRSLQSATES ILVDDMVNSL QSLIFETNSI ITNVVSIGNL 236
slp1_caeel ECLSLSSPQI AARLLINGDW THLHKCAVAL NQLIDMCRGR SS...SSNQR PMSIYARKGKW 191

hbra       ARRLADLLLR MRKEIDAEES SGLTGLSFRG LLPSSSIESL IIIDREVDFG TPLLTQLTYE 151
afvac      ARRLADLLLR MRKELDAEES SGLRGPSARG LLPSASTESL IIIDRMVDFG TPLLTQLTYE 151
ratvac     ARQVANMMVR MKREFTGSQN SVFPVF....  .......DNL LLIDRNVDLL TPLASQLTYE 247
slp1_yeast SKRCSHLLKK RIDEHQTEND LFIKGTLYGE RTNCGLEMDL IILERNTDPI TPLLTQLTYA 296
slp1_caeel ASDVAKMMGK IRNSAEADS.  ...MTKNLDP IEGLLKINRI VLIDRWMDPL TPMLSQLTFY 247
```

FIG._2A

```
hbra   GLIDELVGIK HNQADIDTTI AGASSTPQAQ ESSKASQQA. KQGQKRKIQL D.SSDQLFSQ 209
afvac  GLIDEFVGIK NNQADVDTAI VGANSVPQAQ ESSKAPQQTL KQGQKRKIQL D.SSDQLFSQ 210
ratvac GLIDEIYGIQ NSYVK....L PPEKFAPKKQ GGGGGKDLP. ..TEAKKLQL N.SAEELYAE 299
slp1_yeast GIILDDLYEFN SG..... ..... ..... IKIKEKDMNF NYKEDKIWND 328
slp1_caeel GLLDEIYGI. ..... .GMVNSVKVP EMEFKNEKDG DPFQEKEVYL ...IDEVYHR 292

hbra   LRDANFAIVG DILNKVARRL ETDYESRHTA KTTTELREFV NKLPSYQLEH QSLRVHTNLA 269
afvac  VRDANFAIVG DILNKVARRL ESEYETRHAA KTASELREFV NKLPAYQLEH QSLRVHTNLA 270
ratvac IRDKNFNAVG SVLSKKAKII SAAFEERHNA KTVGEIKQFV SQLPHMQAAR GSLANHTSIA 359
slp1_yeast LKFLNFGSIG PQLNKLAKEL QTQYDTRHKA ESVHEIKEFV DSLGSLQQRQ AFLKNHTTLS 388
slp1_caeel LKHSHINAVS IEASKVLAEI RDDEQFDRDK MSVAEYSVLV KKMPKIINRK KMIEVHMRLA 352

hbra   EEIMKNTRSD T...FRKILE VQQNDAAGAD PTYQHPLIEE LIAR.....D IPLKTILRLL 321
afvac  QEIMRNTRSD I...FRKVLE VQQNNAAGTD PTYQHDTIEE LIAR.....D VPLKTVLRLL 322
ratvac ELIKDVTTSE D...FFDKLT VEQEFMSGID TDKVNNYIED CIAQ.....K HPLIKVLRLV 411
slp1_yeast SDVLKVVETE EYGSFNKILE LELEILMG.. .NTLNNDIED IILELQYQYE VDQKKILRLI 445
slp1_caeel EMI....QSH VYCKQSDSIK LERDLLEYSD SDKAIPLIED LIFD.....A SPLNAVLRLI 403

hbra   CLESCMSGGL RPKDLESFKR QVVHAYGHQH LLTFSALEKM ELLQPRSSAT TMLIPGTGTQ 381
afvac  CLESCMSGGL RSRDLENFKK QIVHAYGHQH ILTFSALEKM ELLQPRSSAA TMLIPTAGAQ 382
ratvac CLQSMCNSGL KQKVLDYYKR EILQTYGYEH ILTLNNLEKA GLLK..... .....AQ 457
slp1_yeast CLLSLCKNSL REKDYEYLRT FMIDSWGIEK CFQLESLAEL GFFTSKTGKT DLHI.TTSKS 504
slp1_caeel SVHSLTCGGL KPSVLQHYRR IVNQSYGSSA LNKVLKMQKM GLIREKGGGG KMQCEYA... 460

hbra   TGSKTNYAYF RKNLRLVVEE V..... SEKEPEDIAY VYSGFAPLSI RLVQCVLQKS 432
afvac  PGTKTNYNYL RKNLRLLVEE V..... SEEDPNDIAY VYSAFAPLSI RLVQCVL... 430
ratvac TGGRNNYPTI RKTLRLWMDD V..... NEQNPTDISY VYSGYAPLSV RLAQLL.... 504
slp1_yeast TRLQKEYRYI SQWFNTVPIE DEHAADKITN ENDDFSEATF AYSGVVPLTM RLVQMLYDRS 564
slp1_caeel ...QMMFQQM KKNHDMLPEE F..... SEAKLDDMAY AYSGFSPLLC KMLE..... 502
```

FIG._2B

```
hbra   YVMSLMKGGP AAHANTASPG WLGYEDVVKS ARGSTFSIVQ KGDDKAVRAR QTLSGNNAAK 492
afvac  .......... .......... .......... .......... .......... .......... 430
ratvac .......... ......SRPG WRSIEEVLRI LPGPHFEERQ PLPTGVQKKR QP....GENR 544
slp1_yeast ILFHNYSSQQ PFILSREPRV SQTEDLIEQL YGDSHAIEES IWVPGTITKK INASIKSNNR 624
slp1_caeel .......... .......... .......... ..EGDRVKWV GWPKTVIGDK SDLIAERDGR 530

hbra   .......... .....TVYVF FLGGITFTEI AALRFIA... AQEAPRRNIV ICTTGIINGD 534
afvac  .......... .......... .......... .......... .......... .......... 430
ratvac .......... .....VTLVF FLGGVTFAEI AALRFLS... QLEDGGTEYV IATTKLINGS 586
slp1_yeast RSIDGSNGTF HAAEDIALVV FLGGVTMGEI AIMKHLQKIL GKKGINKRFI IIADGLINGT 684
slp1_caeel .......... .....GTCVF VIGGLTRSEL AIIR...... ..ENLPNVAL ITTSALITGD 567

hbra   RMMDAALEKG GFALTES  551
afvac  .......... .......  430
ratvac SWLEALMEKP F*.....  597
slp1_yeast RIMNSIS... .......  691
slp1_caeel KLLNNITN.. .......  575
```

FIG._2C

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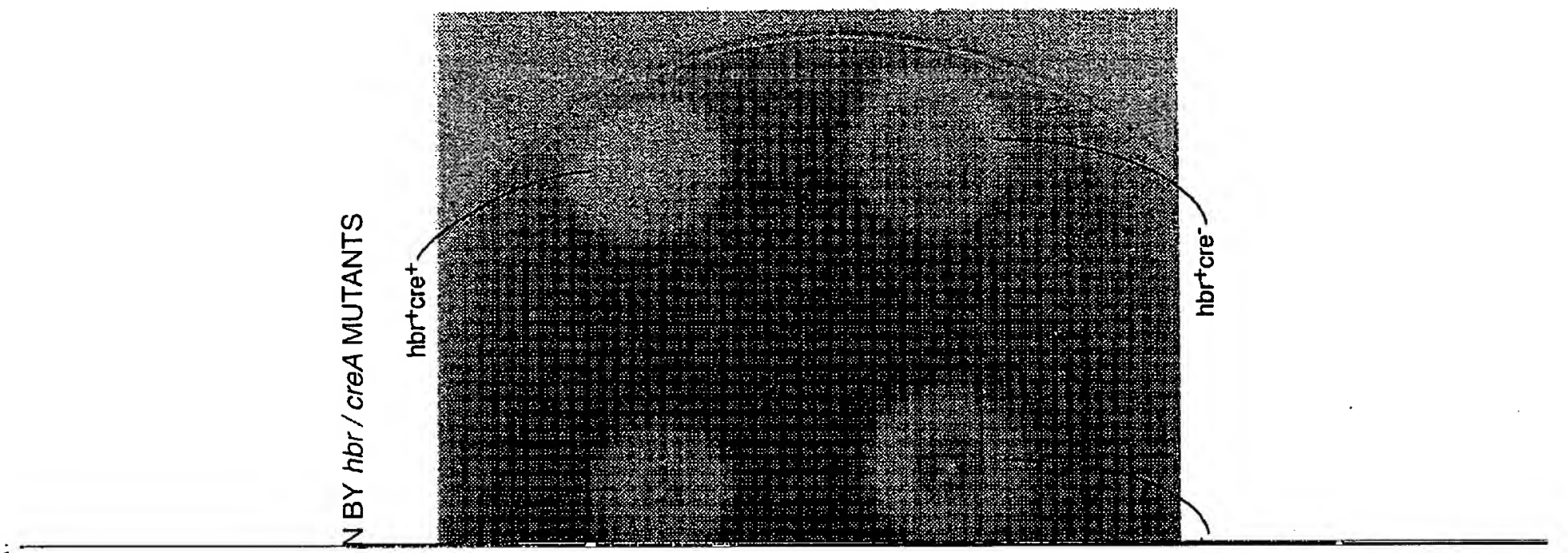
SUBSTITUTE SHEET (RULE 26)


hbr+cre+
hbr+cre-
N BY hbr / creA MUTANTS

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/07615

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C07K14/38 C12P21/02 C12N1/15 //(C12N1/15, C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EMBL, EPO-Internal, STRAND, CHEM ABS Data, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ERJAVEC Z ET AL: "Applicability of random primer R143 for determination of Aspergillus fumigatus DNA." JOURNAL OF MEDICAL & VETERINARY MYCOLOGY, vol. 35, no. 6, November 1997 (1997-11), pages 399-403, XP000929979 ISSN: 0268-1218 abstract page 400, right-hand column, paragraph 2 -& DATABASE EMBL 'Online! Accession AF004837, 28 June 1997 (1997-06-28) ERJAVEC Z ET AL: "Aspergillus fumigatus putative vacuolar protein sorting homolog gene, partial cds." XP002144970 72.9% identity in 1362 BP overlap with SEQ ID NO 1 --- -/--	4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2000	28/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

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INTERNATIONAL SEARCH REPORT

Inter...onal Application No
PCT/US 00/07615

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! Accession AI212286, 20 October 1998 (1998-10-20) KUPFER D ET AL: "x1f08a1.r1 Aspergillus nidulans 24hr asexual developmental and vegetative cDNA lambda zap library Emericella nidulans cDNA clone x1f08a1 5', mRNA sequence" XP002144971 98.8% identity in 325 BP overlap with SEQ ID NO 1 ---	4
X	DATABASE EMBL 'Online! Accession AA784458, 8 February 1998 (1998-02-08) KUPFER D ET AL: "e4b02a1.r1 Aspergillus nidulans 24hr asexual developmental and vegetative cDNA lambda zap library Emericella nidulans cDNA clone e4b02a1 5', mRNA sequence" XP002144972 99.1% identity in 318 BP overlap with SEQ ID NO 1 ---	4
P,X	MEMMOTT S ET AL: "Abstract of Poster: 339. Morphological and genetic characterization of Hbr-2, a hyperbranching mutant of Aspergillus nidulans" 20TH FUNGAL GENETICS CONFERENCE, 'Online! 24 - 28 March 1999, XP002144968 Retrieved from the Internet: <URL:http://www.fgsc.net/asilo99/posterabs3.htm> 'retrieved on 2000-08-11! the whole document ---	1-9, 22-29
X	YARDEN O ET AL: "COT-1 A GENE REQUIRED FOR HYPHAL ELONGATION IN NEUROSPORA-CRASSA ENCODES A PROTEIN KINASE" EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 11, no. 6, 1992, pages 2159-2166, XP002144969 ISSN: 0261-4189 cited in the application the whole document --- -/--	22-25, 27,28

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INTERNATIONAL SEARCH REPORT

Inter...onal Application No
PCT/US 00/07615

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCGOLDRICK C A ET AL: "MYOA OF ASPERGILLUS NIDULANS ENCODES AN ESSENTIAL MYOSIN I REQUIRED FOR SECRETION AND POLARIZED GROWTH" THE JOURNAL OF CELL BIOLOGY,US,ROCKEFELLER UNIVERSITY PRESS, vol. 128, no. 4, 1 February 1995 (1995-02-01), pages 577-587, XP000530233 ISSN: 0021-9525 abstract	22-25
X	WO 97 26330 A (NOVO NORDISK BIOTECH INC) 24 July 1997 (1997-07-24) abstract see examples see claims	27-29

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ..tional Application No
PCT/US 00/07615

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WO 9726330 A	24-07-1997	AU 1750697 A	11-08-1997
		CN 1219971 A	16-06-1999
		EP 0877801 A	18-11-1998
		JP 2000503537 T	28-03-2000
		US 6066493 A	23-05-2000